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Time Course of Wound Healing

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ABSTRACT: Wound healing is a special kind of inflammation. Undisturbed wound healing is subject to a fixed time schedule of biochemical and cellular events. It is virtually impossible to deal with the time course of wound healing without describing the cellular and non-cellular events involved. The activity and mode of cell action after injury are coordinated by spatial and chronological factors, as well as by different mediators and cell–cell interacting signals. During wound healing the sequence of different signals and message substances, such as mediators of inflammation, fulfill a key function in wound repair. The report describes the time course of healing and the control of cellular events by different mediators and cell interactions. Emphasis is placed on temporal aspects, including the various signals leading to typical cellular events in wound healing.

PHASES OF WOUND REPAIR

Regardless of the type of wound, we make a distinction between three characteristic phases of wound healing. These phases differ in terms of how long they last, but they also partially overlap:

1. The inflammatory or exudative phase. When a tissue is acutely damaged and a vessel is opened, allowing blood and lymph to escape into the wound cavity, the first local reaction of the wounded organism is to activate the clotting system (clotting phase) [1]. It can be regarded as the initial "launching" of the healing process of a wound [2–5]. The further course of wound healing is primarily activated by the signals that are given off during this phase [6–10]. The migration of inflammatory cells (polymorphonuclear neutrophilic granulocytes, macrophages, lymphocytes, mast cells) into the coagu-

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lum is in the foreground of the healing process. The beginning of this phase is marked by catabolism.

2. The proliferative or regenerative phase is above all characterized by increased fibroblast activity and by the acceleration of cell division, as well as by the proliferation of blood vessels. Granulation tissue is formed. Anabolism marks this phase.
3. The repair phase is characterized by the formation of new connective tissue, the activity of the myofibroblasts (wound contraction), the maturing of collagen, and the reepithelialization of the wound.

The schematic representation of the time course events (Table 1) serves as orientation to the individual cellular events and the factors causing them.

FUNCTIONAL AND PHENOTYPIC CHANGE OF CELLS

The activity of cells and the mode of cell action after injury seems to be coordinated by different signals and physical conditions. The biochemically active substances and physical states represent a kind of communication system on the basis of a cellular and biochemical vocabulary in the organism and fulfill a key function in wound repair.

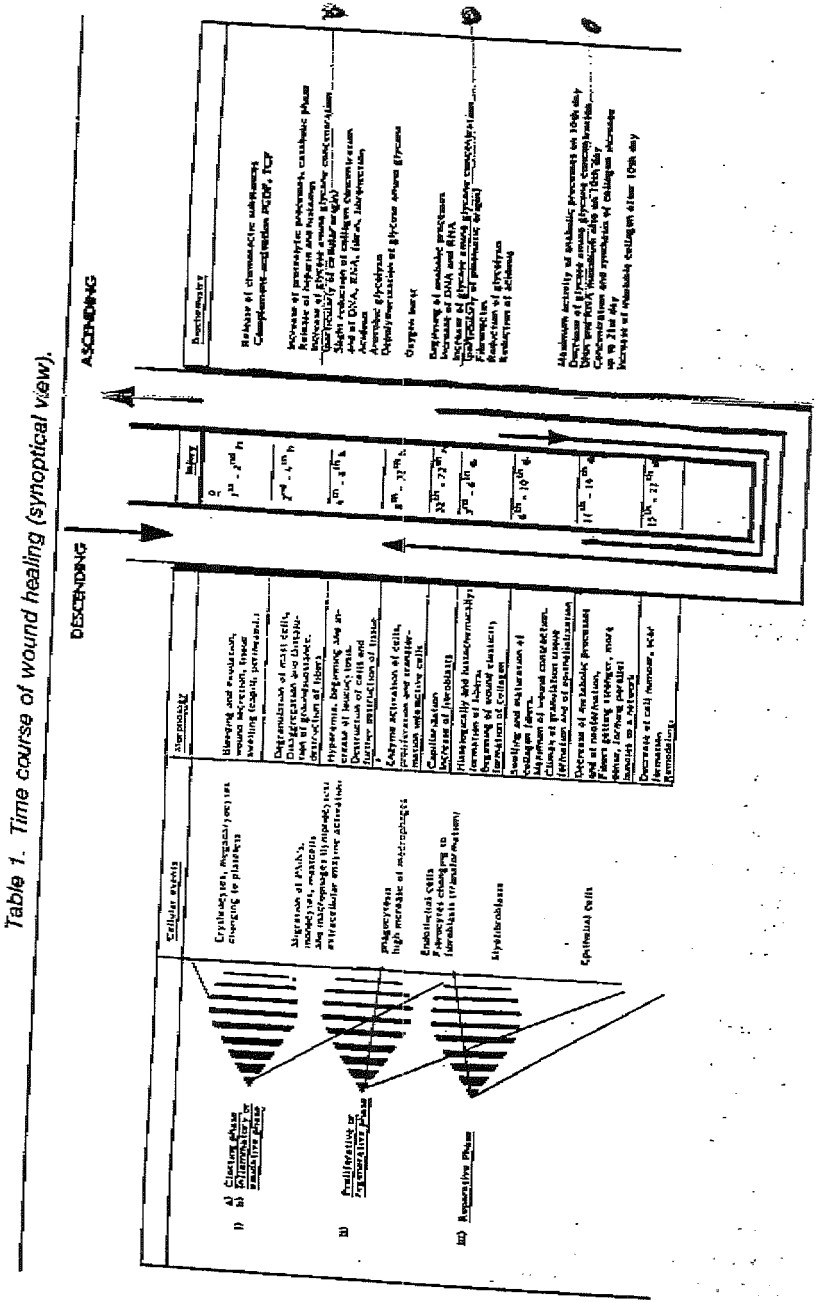
Locomotion and Chemotaxis

Without directional movement of cells, a wound cannot heal. Among the cells that take part in the healing of a wound, a differentiation must be made between those which are found primarily in the flowing blood and only secondarily migrate into the tissue or wound, and those, e.g., fibroblasts and epithelial cells, which participate foremost in the formation of tissue structures. In this context, two basic principles of orientation have to be distinguished:

1. Chemotaxis: the movement of the cells is actuated and made able to maintain its orientation by means of chemotactic mediators. This principle applies mainly to the phagocytes.
2. Contact guidance: the term refers to the movement and orientation of the cells along the guide structures. This type of movement especially pertains to the epithelial cells and fibroblasts.

The organelles and cell structures that make cell movement possible have been studied most intensively on the leukocytes [11,12]. One can assume that the structures and organelles responsible for cell movement are the same for all cells. The difference, however, seems to lie in

Table 1. Time course of wound healing (synoptical view).



the mechanism by which a cell is stimulated to move. The locomotion of the cells suspended in the bloodstream is set off by chemotactic signals.

The cells normally located in cell-and-tissue communities, such as epithelial cells, receive their signal to move when they lose touch with their neighbor cells. They then orient themselves along the way using guide structures. This is known as contact guidance.

Microtubules and microfilaments take part—directly or indirectly—in the formation and maintenance of the cell shapes (cytoskeleton) and in the motility phenomenon. Chemoattractants stimulate the assembly and the organization of microtubules, as well as the localization of microfilaments in the advancing cells.

The repair of small endothelial wounds is an important process by which endothelial cells maintain endothelial integrity. Wong and Gotlieb investigated the repair of endothelial defects under temporal aspects in an *in vitro* wound model system that was used in which precise wounds were made in a confluent endothelial monolayer [13]. The repair process was observed by time-lapse cinemicrophotography. Using fluorescence and immunofluorescence microscopy, the cellular morphological events were correlated with the localization and distribution of actin microfilament bundles and vinculin plaques, and centrosomes and their associated microtubules. Single- to four-cell wounds underwent closure by cell spreading, while wounds seven to nine cells in size closed by initially spreading, which was then followed 1 h after wounding by cell migration. These two processes showed different cytoskeletal patterns. Cell spreading occurred independent of centrosome location. However, centrosome redistribution to the front of the cell occurred as the cells began to elongate and migrate. While the peripheral actin microfilament bundles (i.e., the dense peripheral band) remained intact during cell spreading, they broke down during migration and were associated with a reduction in peripheral vinculin plaque staining. Thus, the major events characterizing the closure of endothelial wounds were precise in nature, followed a specific sequence, and were associated with specific cytoskeletal patterns which most likely were important in maintaining directionality of migration and reducing the adhesion of the cells to their neighbors within the monolayer.

The cells facing the wound underwent retraction after removal of the single cell. Focal cell membrane ruffling with extension of small filopodia into the wound occurred within 5 minutes after wounding. This ruffling became generalized, involving the entire side of the cell abutting upon the wound. Thereafter, the extrusion of the broad flat lamellipodia was observed. The sides of the cell remaining in contact with the monolayer did not show marked ruffling activity.

Circular three- to four-cell wounds underwent closure in a fashion similar to that of single-cell wounds in that extrusion of lamellipodia from all of the cells abutting upon the wound occurred. No cell migration or cell mitosis was observed. Cells immediately behind the first row of cells bordering on the wound did not participate in wound closure.

The removal of seven to nine cells from the confluent closure was followed by retraction of all the cells abutting upon the wound. Within 5 minutes, cell ruffling and the beginning of lamellipodia extrusion was observed. By 30 minutes, broad, flat, lamellipodia had appeared and became prominent over the next 30 minutes. By 60–90 minutes, cell elongation had become apparent and cell migration occurred usually within the next 60 minutes. Wound closure occurred within 90 minutes after the onset of migration. Observations of intact monolayers before wounding did not show any migration.

This study shows that the repair of defects in an *in vitro* endothelial monolayer is a multistep process involving a spreading event and, if necessary, a migration event, each characterized by specific distribution of cytoskeletal systems.

Chemotaxis

Chemotaxis is one of the phagocytes' responses to inflammatory stimuli. It effects the directional movement of these cells into the wound cavity that has been closed by the fibrin network [14,15].

A necessary condition for chemotaxis is tissue alteration, which results in the activation of complement and the secretion of various mediators.

One of the initially important effects is processed by bradykinin and prostaglandins: the vessels enlarge and the permeability of the capillaries and the venules increases. When a vessel is dilated, hemodynamic changes occur whereby the blood elements distribute themselves more towards the wall of the vessel (margination, Figure 1). As Metschnikow already observed, the leukocytes, but not the erythrocytes, attach themselves to the vessel wall and begin to penetrate it [16]. This attachment and the subsequent penetration of the vessel wall take place at every point of the vessel that lies close to the origin of the chemotactic factors.

After a chemical attractant molecule has been linked with its recep-

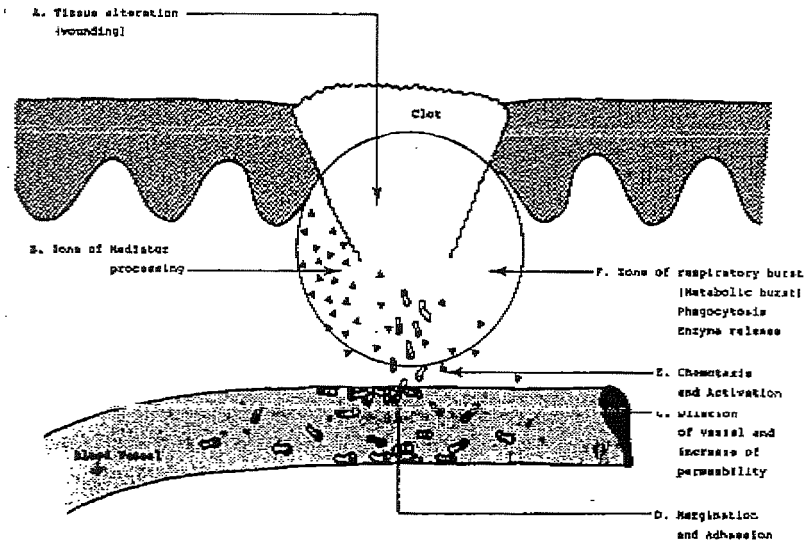


Figure 1. Inflammatory processes following wounding. Letters designate time course of events [107].

tor, the membrane becomes hyperpolarized and oxygen, sodium, and glycose are absorbed by the cell. Potassium is secreted and calcium flows both in and out of the cell, with most of it flowing into the cell [17]. These ion movements in the membrane cause the activation of the membrane-linked phospholipases and the secretion of arachidonic acid, prostaglandins, and leukotrienes. The cyclical AMP increases for a short time, actin filaments, which together with myosin constitute the motor of the cell, are shortened. Finally, the cell orients itself towards the maximum chemotactic stimulus and cell migration begins. A just recently discovered calcium-binding protein, gelsolin, causes the actin filaments to redistribute themselves before cell migration starts [11,18].

In summary, chemotaxis at the cellular level has three phases:

1. An initial or sensory phase, in which a signal is generated by the interaction of the attractant and its receptor
2. The intermediate phase, in which the signal is processed to the cell's motility elements
3. A terminal or effector phase, in which the motility apparatus (both microtubules and microfilaments) is activated to produce directional migration.

Contact Guidance

Cells that normally live in cell communities (e.g., epidermal cells) do not have to be stimulated in order to move [19]. On the contrary, motility as an expression of cellular instability is a primary feature of any cell that is free and unrestrained. Motion pictures of isolated cells *in vitro* show them in a state of permanent agitation. An epithelial cell completely girded by fellow cells becomes immobilized. Cells at the edge of a wound resume motion for no reason other than that their surface has been deprived of its former contact with fellow cells. Epidermal cells, fibroblasts, and nerve cells attach themselves to structures and let themselves be led blindly along these structures guided solely by contact. This principle of orientation is termed "contact guidance." If contact guidance is lacking, the movement of the cell loses its direction.

In connection with wound healing, it is the directional fibrin strands which are generated when the wound contracts that can serve as a contact guide for this type of cell orientation.

INFLAMMATORY OR EXUDATIVE PHASE

Clotting Phase

The healing of a wound begins with blood clotting. Blood clotting and fibrinolysis take place through the interaction of the vessel wall, the thrombocytes, and plasma factors. As a result of this interaction, the primary closure of the wound—the platelet plug—forms, which is stabilized by fibrin deposits [7]. After the vessel has been repaired the thrombus is dissolved and discharged from the fibrinolytic system in the further course of the wound healing process [20,21].

The molecular biological principles of the plasma clotting process are for the most part known today. Most of the clotting factors are enzymes which, in small concentrations, act as catalyzers to produce a considerable biological effect. These enzymes also include the so-called contact factors, namely, proteases, which become activated when blood comes into contact with foreign surfaces [22].

For wound healing, it is important that thrombin initiate two reactions during the clotting cascade, both of which follow the principle of limited proteolysis: it "activates" fibrinogen and F XIII. When the paired fibrinopeptides are split up, fibrinomonomers are produced, which aggregate via end-to-end and side-to-side accumulation to form fibrin strands. These are then covalently linked by the activated F XIII [23,24].

The beginning of cellular activity during wound healing is marked by the migration of inflammatory cells (after 2–4 hours) and fibroblasts (after 32 hours) into the fibrin plug [25,26]. Hence we can see that certain spatial conditions for the further repair of the wound are set during the early clotting phase: a 3-dimensional fibrin network is formed, which serves as the guide-rail for the migration of fibroblasts [19,27, 28]. At the same time, this 3-dimensional fibrin network forms a kind of closure, which prevents microbes from penetrating the wound.

Platelets

The platelets, which are derived from the megakaryocytes, play an essential role during the clotting phase. Various studies in the last few years have referred to platelet released factors, which hold important functions for the inflammatory phase. Knighton et al. [7] characterized platelets and fibrin as initiators for monocyte migration, fibroplasia, angiogenesis, and collagen synthesis. Rutherford et al. [6] described platelet factors that stimulate fibroblasts to proliferate *in vitro*. Archer et al. [8] showed that a platelet activating factor (PAF-acether) possesses properties of mediators of inflammation, as they are also found in other cells in the inflammatory phase. PAF-acether is an ether-linked analogue of phosphatidylcholine. A synergistic interaction between PAF-acether and prostaglandin E has been established. The PAF can therefore be considered a potential mediator of both acute and persisting inflammation in man. It thus belongs to the numerous signal substances which initiate the inflammatory phase after the bleeding has stopped and cause the polymorphonuclear leukocytes, macrophages, and mast cells to move out of the tissue and the vessels and into the wound cavity.

Polypeptides such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) markedly potentiate tissue repair *in vivo* [29]. TGF-β transiently attracts fibroblasts into the wound and may stimulate collagen synthesis directly. In contrast, PDGF is a more potent chemoattractant for wound macrophages and fibroblasts and may stimulate these cells to express endogenous growth factors, including TGF-β, which in turn directly stimulate new collagen synthesis and sustained enhancement of wound healing over a more prolonged period of time.

Time Analysis of Cellular Influx into Wounds

By using polypeptides such as PDGF and TGF-β the time course of

Table 2. Quantitative analysis of cellular influx in PDGF-BB or TGF-β1-treated wounds [29].

Days After Wounding	Growth Factor	Difference In Cellularity	Difference Granulation Tissue	Predominant Cell Type
1–2	TGF-β1	+0.08 ± 0.25		Neutrophil, macrophage
3–5	TGF-β1	+0.64 ± 0.22		Macrophage, fibroblast
7–10	TGF-β1	+0.12 ± 0.26		Fibroblast
14	TGF-β1	+0.14 ± 0.21	+0.18 ± 0.38	Fibroblast
21	TGF-β1	+0.18 ± 0.26	+0.17 ± 0.29	Fibroblast
14–21	PDGF-BB	+0.70 ± 0.24	+0.75 ± 0.26	Fibroblast
28–49	PDGF-BB	−0.36 ± 0.24	+0.36 ± 0.37	Fibroblast

the migration of macrophages and fibroblasts into the wound can be studied. Pierce et al. [29] analyzed the cellular influx into TGF-β1-treated wounds and quantitatively compared it with matched, paired control wounds (Table 2). Increased macrophage and fibroblast influx occurred within 3–5 d of wounding in TGF-β1-treated wounds. This enhancement of cell migration was qualitatively and quantitatively decreased relative to the enhancement found previously in recombinant human PDGFB chain homodimers (PDGF-BB) treated wounds. PDGF-BB induced a large increase in the influx of neutrophils on days 1 and 2 and of macrophages and fibroblasts on days 3–5. The PDGF-BB enhancement of cell migration was substantially above the cellular influxes induced in TGF-β1-treated wounds. The influx of cells in response to PDGF-BB *in vivo* correlated with chemotactic responses *in vitro*. Wounds treated with lower concentrations of TGF-β1 demonstrated less of a cellular influx, in contrast to the more potent effects observed at lower concentrations of TGF-β1 in the chemotaxis assay. TGF-β1 thus is nearly 40,000-fold more potent on a mole.

Complement

In the clotting phase the complement system is also biologically significant. After injury complement activation takes place, i.e., the enzymatic cleavage of C3 and C5, the resulting decomposition products, C3a, C3b, C5a, and C5b, perform the most important biological functions of the complement system [10,30,31]. For our purposes it is particularly important to determine that C5a and C3a under special circumstances prompt the neutrophilic granulocytes, monocytes, and macrophages to migrate. This process is known as chemotaxis. It is one

of the factors responsible for the leukocytes' infiltration of the fibrin plug. The complement factors not only stimulate the neutrophilic granulocytes to perform leukotaxis, but also to secrete lysosomal enzymes. C3a and C5a stimulate the mast cells and basophilic granulocytes to release, along with histamine, the platelet activating factor, which in turn triggers off the releasing reactions of platelets [8,32–34].

Inflammatory Response

Tissue injury after wounding and clotting is followed by an inflammatory response, which is characterized by a relatively rapid accumulation of polymorphonuclear neutrophilic leukocytes, lymphocytes, and macrophages at the site of the injury (Figure 2). This migration of inflammatory cells into the site of an injury is a mark of the early phase of inflammatory response and the exudative phase. It is a necessary condition for the normal course of wound healing.

The platelet derived factors and activated tissue complement factors attract leukocytes. In this early phase polymorphs, monocytes, and lymphocytes entering the wound appear to be devoted primarily to preventing infection.

The Macrophage

The macrophage is a long-lived cell with considerable synthesizing

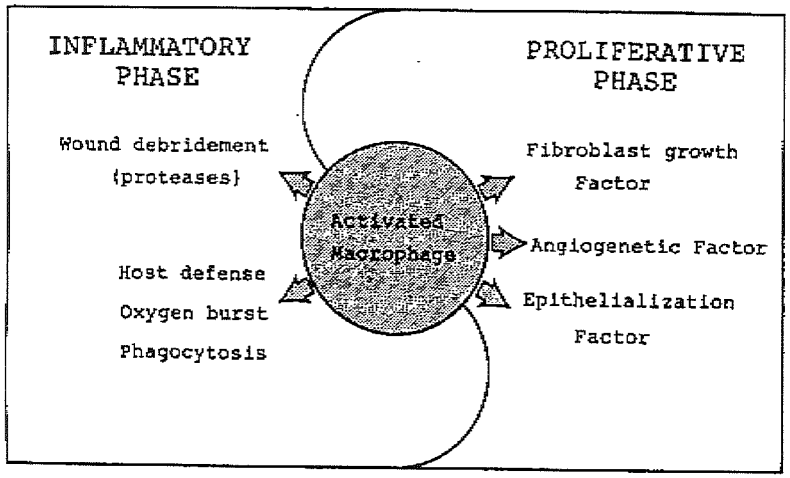


Figure 2. Function of macrophages in wound healing [107].

abilities and with a remarkable potential for functional differentiation. In addition to its well known phagocyte function, the macrophage carries out important secretory tasks for purposes of wound healing [34]. The macrophage has a large Golgi apparatus. A highly developed, rough endoplasmic reticulum and numerous mitochondria are indications that the macrophage is a cell with high synthetic and metabolic activity [35,36].

As we know from the work of Leibovich et al. [37], the systemic administration of hydrocortisone results in a monocytopenia, while the local administration of anti-macrophage serum impairs the phagocytic activity of cells. Due to the reduction of the number of circulating macrophages and to their phagocytic capacity, both wound debridement and migration, as well as synthetic activity of fibroblasts, are conspicuously delayed. This delay again demonstrates that, in contrast to neutrophils, macrophages are essential to wound debridement and fibroblast activity, and hence to healing.

To understand the role of T cells in postinjury fibroplasia, Barbul et al. studied wound healing in congenitally athymic nude mice that lack a normally developed T cell system. The data suggest that T cells play a role in wound healing: an early stimulatory role on macrophages [38].

Macrophages contribute significantly to the normal healing of wounds. Progress in wound repair is dependent on factors provided by activated macrophages [39,40]. Cultured macrophage-conditioned media induce an increase in mesothelial replication. The mitogenic activity present in both—wound exudates and macrophage-conditioned media—is increased by dialysis and diminished by heating at 80°C for 1 h. The putative mesothelial mitogenic factor in the supernatant of wound exudates and macrophage cell cultures has a molecular size greater than 7,000 daltons and is stable after mild heating (60°C, 1 h). It is postulated that exudate macrophages secrete mitogenic factor(s) which stimulate mesothelial proliferation and initiate healing. It is unclear whether the postulated mitogenic factor(s) derived from cultured macrophages and also present in wound exudates are similar [41].

Another substance that may be responsible for the above observations is the polypeptide interleukin-1. This molecule is secreted by activated macrophages, is stable after mild heating (56°C, 30 m), has a molecular size of 14,000–50,000 daltons, is susceptible to protease, and is capable of stimulating DNA synthesis of fibroblasts.

Macrophages secrete a mitogenic factor that induces proliferation of mesothelial cells adjacent to the wound and on the opposing surface. The proliferative response of neighboring mesothelial cells is at its

highest level in 2 days after the injury, coinciding with the time macrophages are at their greatest concentration in the wound exudate.

Angiogenetic and fibroblast growth factors are released by activated macrophages [40]. Similar proteins are known to produce hyperplasia of the epithelium and macrophage factors probably also account for the stimulus to epithelialize. In short, the macrophage seems to encode the events of injury and translate them into a variety of repair signals (Figure 2).

In conclusion, one can say that the substances released from activated platelets and macrophages during the inflammatory and exudative phase constitute the basic prerequisite for the further course of healing.

Proliferative/Regenerative Phase

Cell proliferation marks the next phase of wound healing. In this phase of wound healing, wound edema plays a special role. It has been shown that the increase in tissue fluid, along with the activation of the histiocytes, causes the transformation of fibrocytes to fibroblasts [20,42].

The Fibroblast

Fibrocytes already undergo a phenotypic change in the edema phase and acquire the ability to transform into active fibroblasts by developing organelles essential to collagen synthesis and secretion.

Immediately after the blood clot develops, generation of highly vascularized granulation tissue, the basis for effective epithelialization, begins. Granulation tissue is formed from capillary buds, new capillaries, and fibroblasts. Bouisson et al. conducted an electron microscopic study to demonstrate the origin and the development of fibroblasts forming granulation tissue [43]. The results indicate that fibroblasts originate from resting fibrocytes in the wound margins. These resting fibrocytes first become undifferentiated mesenchymal cells termed "X" cells. The "X" cells then multiply, migrate, and invade the wound defect in approximately 3 days, transforming into highly active fibroblasts. The active fibroblasts are endowed with the capacity of further transformation to fibroclasts and myofibroblasts. The latter two cell populations then effectively cause remodeling of newly formed tissue and contraction of wound margins. Local fibrinolysis begins and at the same time, new capillaries are being formed.

Through their fibrinolytic potential, endothelial cells effect a dissolution of the fibrinous network [44–46]. In addition to these catabolic processes, the transition to an anabolic metabolism also begins [9,20].

The restoration of tissue continuity following injury and the strengthening of ensuing repair tissue depends largely, if not entirely, on the resulting fibroplasia and the activity of the fibroblasts. The fibroblasts proliferate and migrate during the entire healing process. The connection between fibroblast proliferation and a macrophage-dependent factor has already been mentioned.

Analysis of Time Course of Fibroblast TGF-β Synthesis in Wounds

Pierce et al. stated that macrophages are not prominent in tissue sections of glucocorticoid-treated wounds after 7–10 d, suggesting that PDGF might act at the level of the fibroblasts in 7 to 10-day wounds over and above its influence on the macrophage early in the wound-healing process (see also p. 14). Sections from 2, 4, and 7 d PDGF-BB-treated and paired control wounds were analyzed with a monospecific anti-TGF-β antiserum. The results indicated that PDGF was capable of inducing increased intracellular TGF-β levels *in vivo*, both in the macrophage and in the fibroblast. TGF-β observed in wound fibroblasts can arise from a direct stimulatory action by PDGF. The increase in procollagen type I observed in these experiments may arise from the subsequent autocrine stimulatory influence of newly synthesized TGF-β1 [29].

It appears that fibroblast migration precedes their proliferation [47]. The importance of collagen for fibroblast motility has been demonstrated by showing chemotactic attraction of fibroblasts to types I, II, and III collagen-derived peptides and the binding of chemotactic collagen-derived peptides to fibroblasts [48].

While the blood clot is being lysed in the late exudative and in the early proliferative phase, amino acids are produced, which serve as a substrate for the new fibroblasts [9,20]. Pohl et al. [25] observed an increase in fibroblast proliferation by thrombin and fibrin *in vitro*. These findings show that in fibrin clots, fibroblasts spread and reproduce.

Bucknal [49] emphasized the connection between impaired healing in infected wounds and the role of fibroblasts. The cause of impaired healing proved to be decreased fibroblast concentration and activity.

The regeneration of protein begins to increase after the fibroblasts form cell trails, which correspond to the later texture [50]. The fibroblasts passing through the wound produce collagen fibrillae. These fibrillae contract to diminish the size of the wound and build intercellular bridges that increase the tensile strength of the tissue [51,52].

Harris et al. [51] described fibroblast traction as a mechanism for collagen morphogenesis. The authors examined the effects of cellular traction on re-precipitated collagen matrices. They found that the traction among the various fibrocyte types differs and that paradoxically, it is weakest in the most mobile and invasive cells. Untransformed fibroblasts exert forces much stronger than those actually needed for locomotion. This strong traction dramatically distorts collagen gels and creates patterns similar to tendons and organ capsules. This morphogenetic rearrangement of extracellular matrices seems to be the primary function of fibroblast traction and explains its excessive strength [53].

The Myofibroblast

Myofibroblasts appear in the wound about 6 to 10 days after injury. Wound contraction produced essentially by myofibroblasts is one basic mechanism of wound closure [43]. Under certain circumstances, the fibroblasts can be differentiated into a cell type that is structurally and functionally similar to smooth muscle [54–56]. These modified fibroblasts (myofibroblasts) are the cellular agent of wound contraction [20,57,58]. During healing, the wound surface becomes smaller as the edges move together. This wound diminishment reduces the regeneration of connective tissue and epithelium necessary for healing by 50–99%, depending on the part of the body. The wound contraction and the shrinkage due to scarring reduce the diameter of a well-granulated wound by 1–2 mm per day [59].

The mitotic activity of the fibroblasts ends with the beginning of collagen fiber formation on about day 10 to 15. The gradual transformation to scar tissue takes place as the number of collagen fibers increases, until a balance between synthesis and lysis is reached after approximately three weeks [52].

EPITHELIALIZATION

Epithelialization usually starts from the edges of the wound, unless the wound is a shallow epidermal one and the basement membrane has been preserved [60]. In this case, the entire area of the wound can be re-epithelialized by mitosis from the remaining cells of the basement layer.

During the epithelialization phase, the epidermal cells undergo a number of phenotypical changes: the desmosomes, which guarantee that the epidermal cells team up together, are dissolved and peripheral

cytoplasmatic actin filaments are formed, which is another condition for epidermal cell motility. The tonofilaments retract within the cells, without which the epidermal cells cannot move [61,62].

Re-epithelialization can be divided into three stages:

1. Migration–active epidermal cell movement (AECM)
2. Replacement of the destroyed cells by mitotic activity
3. Maturation of the newly formed cells

Active Epithelial Cell Movement (AECM)

In man and in higher mammals, AECM does not occur before 24 hours after the injury. AECM is very important for the initial stage of epidermal wound healing. Smaller defects close, even without a subsequent increase in mitotic activity [20,63,64]. This migration is independent of mitotic activity. The rate of migration, however, is variable and depends on the same conditions as those which encourage or inhibit mitosis. It is not clear what causes cells to migrate or what attracts them to make them want to move towards the wound. As Silver pointed out, it is feasible that they move along electrical gradients, since the latter develop across the junctions of normal and injured tissue [65].

Another possible reason for epithelial cell migration is the breaking up of the cell community on the free edge of the wound. In other words, when the cell senses the loss of "contact with its neighbor," it receives this as a signal for phenotypical transformation, which finally leads to migration and proliferation. This phenomenon is known in the literature as the "free edge effect" [66,67].

Winter showed that the individual epidermal cells do not move more than 2–3 cell lengths from their original position. From this observation he concluded that the new epidermis is formed step by step by implantation of epidermal cells on the wound surface. In other words, when a wound re-epithelializes, the epidermal cells close the wound by forming a kind of chain: the first migrating cell implants itself, the following cell "climbs over" it and, in turn, implants itself to be climbed over by the next cell, and so on. This theory of epidermal cell migration is called "the leap frog hypothesis" [67].

Investigations carried out with precursors of H^3-thymidine-labeled nucleic acid have shown that only the cells of the basal layer are capable of synthesizing DNA and hence of dividing [47,68,69]. The cells move from the basal layer at a speed specific for the particular tissue, while at the same time the cells are transformed into corneocytes. By labeling the cells with radioactive thymidine, they can be closely fol-

lowed as they migrate [70]. In injured pig skin, which most resembles human skin, the cells migrate at a speed of 21 μm per hour [66]. It has been proved that some of the cells formed in the basal layer remain in this layer, while the others move up to the surface.

Following a small, shallow abrasion, mitotic activity may be confined to a distance of 2–3 mm from the edge of the wound, while in more extensive injuries cell division may be as far as 6 mm from the wound [71]. The rate of division is determined by the local cellular environment; it is slowest in dry conditions where the oxygen supply is limited.

About 12–48 hours after wounding, an increase in the mitotic activity of the epidermal cells can be observed [67]. Epidermal wounding is followed by a regenerative response consisting of a burst of proliferative activity seen about 3 days after the tissue damage. Basal cell subpopulations may respond differently and selectively during the wound healing process. Jensen and Bohund investigated the behavior of early keratinization *in vitro* [72]. One-month old primary cultures were subjected to different treatments to strip off the suprabasal cell layers. Before stripping, the cultures covered 75% or more of the culture surface and showed extensive multilayering and keratinization. Stripping was initially attempted by incubating in NH_4Cl and β-mercaptoethanol. However, this treatment apparently caused irreversible damage to the cells, as they were unable to reestablish growth and differentiation after being refed with normal medium. In contrast, when cultures were incubated in Ca^{2+}-free medium for 72 h, this resulted in a reproducible and selective stripping of all suprabasal layers, leaving a monolayer of basal-like cells.

Morphological Changes After Refeeding with Normal Ca^{2+} Medium

When the cultures were refed with normal Ca^{2+} medium, a reproducible series of morphological changes took place. During the first 24 h, the cultures consisted of a monolayer of basal-like cells that appeared somewhat retracted with wide intercellular spaces. No mitotic activity was observed. By 24 h, the cultures had started to become heteromorphic. During the next 2 to 3 days, a burst of mitotic activity occurred, which peaked at 72 to 96 h after stripping. At 96 h poststripping, there was a gradual decrease in mitotic activity as the keratinization proceeded. Seven days after stripping, keratinization and desquamation were extensive and the culture morphology was quite similar to that observed immediately before stripping [72].

The end stage of re-epithelialization is the development of a mature corneous layer. Krawczyk et al. [73] made blisters on hairless rats and

observed re-epithelialization with the electron microscope. After 24 hours they could already see intracellular keratinosomes.

During the reepithelialization of a wound, in which the basement membrane is destroyed, the epithelial cells migrate on a temporary "basement membrane" consisting of fibronectin [75,76], type V collagen [74], and fibrin [77]. Keratinocytes are able to synthesize fibronectin and therefore virtually provide their own guide-structures. The plasminogen activators are the substances that enable the epithelial cells to follow a route through the tissue just below the zone of inflammatory cells.

Not until the epithelial cell migration has been completed is the final basement membrane of type IV collagen and laminin formed [75,78]. The basement membrane follows behind the epithelial cells from the edge of the wound. Reepithelialization ends with the formation of hemidesmosomes and with the adhesion of the epithelial cells to the basement membrane [79].

Epidermal Growth Factor (EGF)

Recent research on the mechanisms of action of the epidermal growth factor (EGF) has been productive. In 1962, Cohen isolated EGF from the submaxillary gland in mice [80]. Meanwhile, different fractions of EGF-character (EGF-precursor, EGF-like peptides) have been observed [81]. Some authors suggest that EGF, alpha-transforming growth factor (alpha-TGF), a T cell growth factor (Interleukin 2) may make up a "gene family" [81,82].

To date, the best studied effects of EGF are its ability to increase proliferation, to differentiate, and even to repair various epithelia, including the skin [83–86]. Specific EGF receptors are found on both epithelial and nonepithelial cells. *In vitro* nonepithelial cells respond to EGF.

Experimental studies in animals have demonstrated that the topical application of epidermal growth factor accelerates the rate of epidermal regeneration of partial thickness wounds and second degree burns. Treatment with epidermal growth factor significantly decreased the average length of time to 25% and 50% healing by approximately one day and 75% and 100% healing by approximately 1.5 days [87]. Epidermal growth factor may stimulate the division of keratinocytes and dermal fibroblasts, both of which have been shown to express receptors for epidermal growth factor [88,89]. It is also possible that exogenous epidermal growth factor stimulates healing indirectly by enhancing the production of other growth factors such as transforming growth factor

alpha or by enhancing the action of growth factors delivered to wounds by platelets or macrophages [89–91]. Regardless of the specific mechanisms, the early, continuous exposure of regenerating cells to high levels of epidermal growth factor promotes epithelialization. This finding supports the concept that growth-promoting factors and their receptors may play important parts in normal healing. Thus, impaired wound healing may result from a local deficiency of growth-promoting factors, an excess of growth-inhibiting factors, or alterations in growth-factor receptors.

In human skin, EGF receptors are found on keratinocytes, especially in the basal layer, sebocytes, smooth muscle cells including arrector pilorum muscles, and myoepithelial cells. To determine the proliferative activity of tissue repair fibroblasts, Fukosawa et al. tested the mitogenic response of tissue repair cells (TRC) to growth factors. Postsurgical (days 2, 5, 7, and 10) tissue repair cells were recovered from the injured peritoneum. Although tissue repair cells consisted of a mixed cell type after 4 days in culture, recovered cells were essentially fibroblasts. These TRC were then pulsed with [3H] thymidine after 4 days in culture. The incorporation of thymidine into postsurgical day 5 TRC increased significantly compared with that of day 2 TRC ($p < 0.05$). Fibroblast growth factor (FGF) and epidermal growth factor (EGF) stimulated the incorporation of thymidine into TRC [92].

Table 3 gives an overview of the number of factors involved in tissue repair.

Scar Formation

Tissue defects are replaced by unspecific connective tissue during scar formation.

The sign of a freshly healed wound's load capacity is its tensile strength. This is mainly dependent on the number of newly formed collagen fibers. During wound healing, it has been suggested, modified fibroblasts rich in actin filaments are responsible for wound contraction. With the use of specific fluorescent probe (NBD-phallacidin), the distribution of actin filaments were compared in normal dermis and in several wound contraction models, including open and burn wounds and full- and thin-thickness skin autografts. Fibroblasts with actin filaments are increased in autografts, particularly at days 15 and 21 after grafting, and are prominent in open and burn wounds. The wound contraction rate is not directly related to the presence of actin-staining fibroblasts. After stabilization of the contraction of open or burn wounds, fibroblasts rich in actin filaments remain. It can be concluded

Table 3. Overview of peptide growth factors with application to wound healing.

Factor	Source	Target Cell	Activities *in vitro*	Effects *in vivo*	*in vivo* Models
EGF/TGF	Epithelium	Fibroblasts	Proliferation	Angiogenesis	Subcutaneous
	Platelets	Epithelium	Contraction	Fibroplasia	Epithelial defects
	Macrophages	Endothelium		Reepithelialization	Burns
		Smooth muscle		Wound contraction	Open wounds
					Corneal stroma
FGF	Fibroblasts	Fibroblasts	Proliferation	Angiogenesis	Subcutaneous
	Endothelium	Endothelium	Differentiation	Fibroplasia	Incised wounds
	Macrophages	Epithelium	Migration	Reepithelialization	Open wounds
	Smooth muscle	Smooth muscle	Matrix synthesis	Wound contraction	Skin grafts
					Corneal stroma
					Epithelial defects
TGF-β	Platelets	Fibroblasts	Matrix synthesis	Angiogenesis	Subcutaneous
	Macrophages	Epithelium	Inhibition	Fibroplasia	Incised wounds
	Lymphocytes	Endothelium	Chemotaxis		Open wounds
	Epithelium	Monocytes	Contraction		
	Fibroblasts	Lymphocytes			
PDGF	Platelets	Fibroblasts	Proliferation	Fibroplasia	Subcutaneous
	Macrophages	Smooth muscle	Matrix synthesis		Incised wounds
	Endothelium	Monocytes	Activation		Open wounds
	Smooth muscle	Neutrophils	Chemotaxis		

(continued)

Table 3. (continued).

Factor	Source	Target Cell	Activities *in vitro*	Effects *in vivo*	*in vivo* Models
NGF	Epithelium Fibroblasts Fibroblasts	Neutrophils Monocytes	Chemotaxis	Wound contraction Inflammation	Subcutaneous Open wounds
TNF	Macrophages Endothelium	Fibroblasts Macrophages Neutrophils Lymphocytes	Inhibition Activation	Angiogenesis Fibrosis Differentiation	Cornea Incised wounds
IL-2	Lymphocytes	Lymphocytes	Proliferation	Fibrosis	Subcutaneous Incised wounds
INF	Lymphocytes	Fibroblasts	Inhibition	Inhibition	Subcutaneous

*The activities and effects listed do not correspond directly to cell types on the same line in the table. Target cells may respond in several ways to a given factor. The list is not exhaustive

Abbreviations
EGF Epidermal Growth Factor
TGF Transforming Growth Factor
FGF Fibroblast Growth Factor
TGFβ Transforming Growth Factor β
PDGF Platelet Derived Growth Factor
NGF Nerve Growth Factor
TNF Tumor Necrosis Factor
IL-2 Interleukin 2
INF Interferon

that the distribution of actin-rich fibroblasts corresponds morphologically to previous areas of necrosis or injury [93].

In culture, migrating cells have diffuse and weak stress fibers, as seen at days 3 and 7 of open and burn wounds, whereas mobile or migrating fibroblasts lack or have diffuse stress fibers. Rich actin-modified fibroblasts with contact specialization such as gap junctions and fibronexus are involved in wound contraction and cell adhesion by cell–cell and cell–extracellular matrix contacts. In addition to these two mechanisms, one can suggest that the adhesion and its "pulling" property of the modified fibroblasts is part of the remodeling of the newly formed extracellular matrix during wound healing [92].

As collagen synthesis sets in, the tensile strength of the wound increases and its peak is reached after about 14 days [94–96]. The collagen content in the wound area gradually begins to return to normal after 4–5 weeks. According to Verzar and Willenegger [97], it can take more than 6 years for the soluble collagen to mature to insoluble collagen fibers. Hydroxyprolin content in the wound area behaves analogous to the hydroxyprolin level in the serum and urine and this is a good criterion for the collagen metabolism: up to roughly the 7th day almost normal hydroxyprolin values are attained, which then increase again as a result of the beginning collagen synthesis [98].

Aging and Wound Healing

Aging affects various aspects of the wound healing process including the multiplication of fibroblasts, the synthesis of collagen, the nature of elastic tissue, the rate of fibroblast growth, and the amount of soluble versus insoluble collagen.

Grove [99] was able to show the age-related differences in the healing of superficial skin wounds in humans. At all stages of repair, older individuals (aged 65–75 years) as a group lagged behind young adults (aged 18–25 years). Chvapil et al [100] discussed the individual phases of the wound repair process to demonstrate the factors that modify the rate and the magnitude of healing, e.g., the effect of age.

Wound healing rate has been used as a biological marker of age in mice. Previous results suggest that altered estrogen or androgen levels or altered sensitivity to these steroids could account at least partially for the slower wound repair in old rats. It has recently been demonstrated that the application of anti-macrophage serum to the experimental wounds in young mice resulted in slower wound healing, similar to that of old mice, during the period of greatest activity – i.e., the first 4 to 5 days after wounding.

The precise age-associated impairment in macrophage function remains to be determined. Preliminary findings have suggested that slower healing rates in aged mice were not due to reduced presence of macrophages in the wound area, although the rate of arrival of macrophages to the wound area was not evaluated. On the other hand, the acceleration of wound healing by macrophages from old mice injected into the wounds of old mice may suggest that macrophage homing capacity may be reduced in advanced age. Thus, some aspect of macrophage function, in addition to migratory capacity, would seem to be affected with age [101]. Carrel and Ebeling [102] have shown that the rate of cellular multiplication of cultured fibroblasts varies inversely with the age of the donor from which the culture medium (plasma) is taken. This work also revealed that in wounds of essentially identical dimensions, the index of cicatrization (an expression of the rate of wound healing) varies inversely with age. Finally, the effect of age in the serum was such that as the age of the donor increased, the amount of the factor inhibiting fibroblast proliferation also increased. Other authors have argued that healing in the young is more rapid than in adults, because fibroplasia begins earlier and proceeds more quickly [103]. These authors proposed that retardation of healing in older individuals results from a combination of an increasing amount of inhibitory substance and an age-related increased autocatalysis. Collagen (both soluble and insoluble) and elastin are markedly affected by the aging process. Soluble collagen decreases with age in both sexes, whereas the insoluble collagen content increases with age. Elastin increases with age, but the quantity of elastic fibers (especially in vessels and skin) decreases.

Other authors [104] observed in an ultrastructural morphometric analysis that numbers of dermal microfibril bundles diminish with age. Fenske and Lorber [105] recently presented a summary of all the results of structural and functional changes of normal aging skin. Events begin later, proceed more slowly, and often do not reach the same level. The ability of the aged to heal so well illustrates, therefore, not that their healing processes are equal to those of the young, but that our healing capacity is far in excess of what is needed [106].

CLOSING REMARKS

This review has focused on the major functional aspects relevant for the time course of wound healing. Cooperation and timing between the different types of cells and soluble mediators in wound healing is very complex and it is difficult to evaluate the relative importance of the various events modifying wound healing.

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book review

Clinical Implant Materials
Proceedings of the 8th European Conference on Biomaterials, Heidelberg, F.R.G., September 7–9, 1989
Edited by G. Heimke, U. Soltesz and A. J. Lee

This excellent book is Volume 9 of the series on "Advances in Biomaterials" by Elsevier Science Publishers B.V. of the Netherlands and Elsevier Science Publishing Co., Inc., P.O. Box 882, Madison Square Station, NY, NY 10159.

The book is a mini-encyclopedia of biomaterials and biomechanics, evidenced by the biomaterials subjects covered: (1) Soft Tissue and Bone, (2) Metals, (3) Polymers, (4) Degradable Polymers, (5) Ceramics, (6) Glasses and Carbon, and (7) Coatings.

Clinical applications covered are: (1) Orthopedics, (2) Vascular Materials, (3) ENT-Surgery, (4) Dentistry, (5) Percutaneous Devices, and (6) Internal Medicine. There is also a section dealing specifically with biomechanics.

The reader will find himself or herself constantly referring to the information contained in this book, since it represents an accurate cross section of this field, presented by a group of international experts in diverse fields.

This reviewer is particularly impressed by the short period of time that elapsed between the time of the conference and publication of this book. The field of biomaterials is a rapidly evolving discipline with discoveries and advances being made at unprecedented speed. A major problem confronting biomaterials scientists is the long time required between submission of an article and its publication.

Publication of papers in scientific journals typically takes about 12 months from submission; in books it may take as long as 2 years. Thus, readers have a knowledge "lag" that in many cases is unacceptably long. It is refreshing to see that Elsevier has found a way to publish such a major book in record short time.